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Effect of Ethanol on the Protein Secondary Structure of the Human Gastric Mucosa, In Vitro

By *Shan-Yang Lin*[1], *Chew-Wun Wu*[2] and *Run-Chu Liang*[1]

[1] *Department of Medical Research and Education*
[2] *Department of Surgery*
Veterans General Hospital-Taipei, Taipei, Republic of China

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Summary: The effect of ethanol on the secondary conformational structure of proteins of the human gastric mucosa was investigated by attenuated total reflection/*Fourier* transform infrared (ATR/FT-IR) spectroscopy. The IR peak intensity and position of each structural component of gastric mucosa was found to change significantly with the ethanol concentration and length of exposure. The peak intensity due to the β-sheet and/or β-turn conformational structure in amide I and II bands of gastric mucosa clearly increased after treatment with ethanol. Moreover, the peak at 1635 cm^{-1} shifted to 1630 cm^{-1} after treatment with 40% ethanol for 3 h, or 80% ethanol for 1 h, and a distinct shoulder also appeared at 1643 cm^{-1}. This shift occurred more rapidly and was more pronounced after exposure of mucosa to 80% ethanol, compared with the effect of 40% ethanol, but the α-helical structure at the amide I and II bands was not influenced by either concentration of ethanol. Ethanol treatment might also transform the secondary structure of amide III in gastric mucosa from an α-helix to a mainly random coil with extensive unfolding. The absorption between 1180 and 980 cm^{-1}, which is assigned to glycoprotein structure, was also reduced after treatment with ethanol. This strongly indicates that ethanol influences the conformation of the lipids and proteins of human gastric mucosa, leading to their deformation.

Introduction

Several alterations in the gastric mucosa of animals and humans, due to disruption of the gastric mucosal barrier, have been studied following the intragastric administration of ethanol (1–3). Ethanol at concentrations > 35% (volume fraction 0.35) may result in focal areas of marked mucosal hyperaemia, necrosis (especially of superficial epithelial cells), oedema, and mucosal and submucosal haemorrhage (4, 5). *Guth* et al. found that 100% ethanol may cause total stasis of blood flow in damaged mucosal areas (6). However, *Lacy* et al. speculated that the small focal ethanol-induced lesions in rats might be easily repaired by rapid epithelial migration under normal physiological conditions (7). These ethanol-induced alterations are also reversible within 3 days if ethanol consumption is stopped (8).

The role of gastric mucus in the prevention of gastric damage remains controversial (9–10), but it is postulated that the gelatinous mucus forms a cap over the damaged region and provides an environment favorable for the process of restitution from disruption (11, 12). Generally, the gastric mucous gel that lines the surface of the mucosa is crucial for epithelial protection. Numerous studies have indicated that ethanol adversely affects the mucus structure, such as changes in the hydrophobicity of gastric mucosa (13), dehydration and denaturation of mucus (14), changes in the thickness of the surface mucus gel (14), and interference with the process of acylation of mucus glycoprotein with fatty acids (15). However, relatively little information is available on the effect of ethanol on the protein secondary conformational structure of the human gastric mucosa.

Application of *Fourier*-transform infrared (FT-IR) spectroscopy for the analysis of non-destructive processes in biological materials has attracted considerable interest as a means of providing useful information on protein structure (16–17). Using an FT-IR spectrometer with attenuated total reflectance (ATR), we have studied the conformation of the stratum corneum of different animal skins, type IV collagen and lens capsule, and rabbit bladder mucosa and serosa before and after vesical outlet obstruction (18–22). In the present study, we also used ATR/FT-IR spectroscopy to investigate the protein secondary conformational structure of the gastric mucosa of isolated human gastric wall after treatment with ethanol, in vitro. The ethanol concentration and the length of exposure necessary to cause structural changes of the human gastric mucosa were also determined.

Materials and Methods

Materials

The human gastric wall was isolated from a male gastric cancer patient after subtotal gastrectomy, and cleaned five times with 500 ml of normal saline. The gastric mucosa on the isolated gastric wall was submitted to pathological examination and confirmed to be normal.

Ethanol treatment

Nine samples of the isolated human gastric wall were directly immersed in 40% aqueous ethanol, and nine similar samples in 80% aqueous ethanol. At the described intervals, three samples were withdrawn and immersed in normal saline several times to remove residual ethanol. These ethanol-treated samples were spread on filter paper to absorb the excess water and the gastric mucosa was then investigated by ATR/FT-IR spectrophotometry.

Infrared spectral analysis

Infra-red spectra of the human gastric mucosa with or without treatment with ethanol were obtained using an FT-IR spectrophotometer equipped with an MCT detector and a zinc selenide ATR prism (Micro FT-IR 200, Jasco, Co., Japan). The spectra were taken at 4 cm^{-1} resolution and generally 200 scans were accumulated to get a reasonable single-noise ratio. The spectra presented in this study were all difference spectra between the spectra of the samples and that of water, calculated by normalizing the intensity of a band near 2120–2150 cm^{-1} due to water (18–22). A baseline correction program was used to correct the curved baseline for all spectra. The peak area ratio of each band to the amide II band was obtained by setting two band central positions, and right and left base positions of each band, then performing an arithmetic operation program, as shown in table 1. The peak area ratio of each peak at 1549 cm^{-1} was also given by setting the same parameters of each peak, as shown in table 2. A display of the peak positions was achieved using a second derivative technique (23).

Statistical analysis

Each ethanol-treated sample was determined and the means and standard deviations (S.D.) were obtained (n = 3). Results among

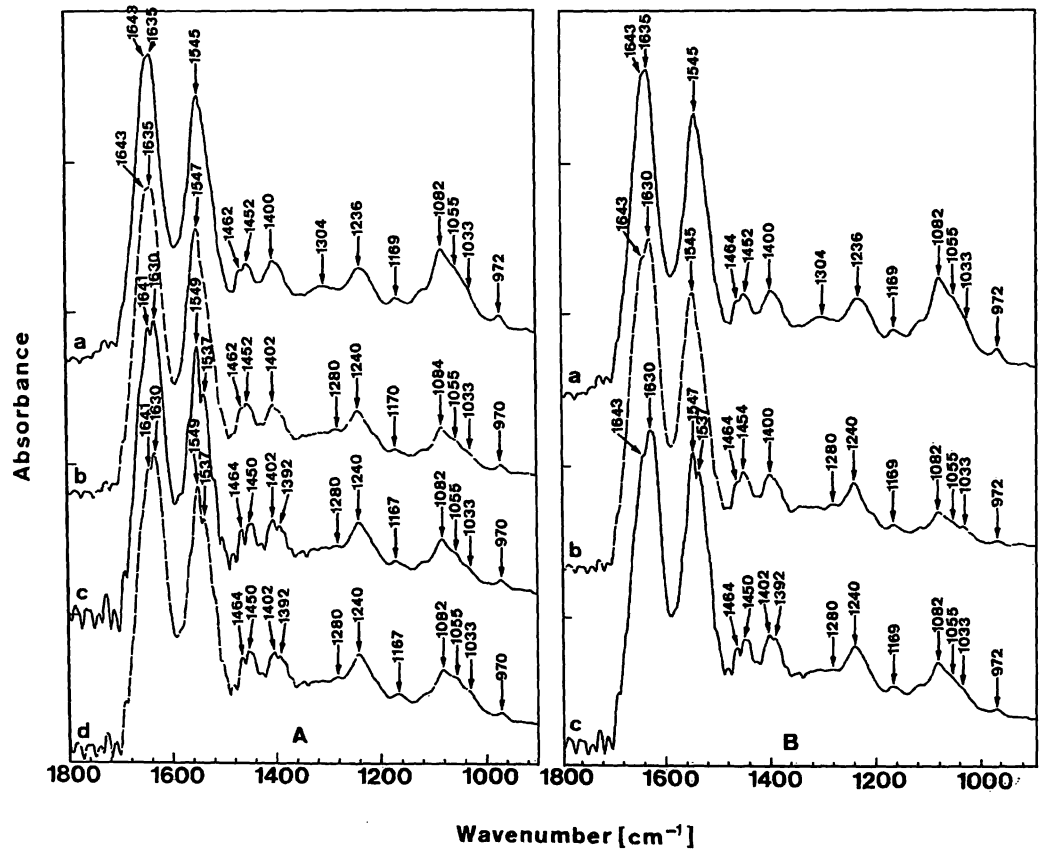

Fig. 1 FT-IR spectra of human gastric mucosa before and after treatment with different concentrations of ethanol

Key: (A) 40% ethanol; (B) 80% ethanol: before treatment with ethanol (a), and after treatment for 1 h (b), 3 h (c) and 8 h (d)

the multiple groups were analysed by one-way analysis of variance (ANOVA) with $p < 0.05$ being taken as the minimal level of significance.

Results

The secondary structure of a protein can be studied from its IR spectrum by use of amide I and II frequencies as key indicators. The amide I band is much more sensitive to changes in the conformation and structure of the protein than the amide II (24). Figure 1 shows the IR spectra of the human gastric mucosa before and after treatment with different concentrations of ethanol. The second-derivative spectra gave precise values for the peak positions of the component bands in the amide I and II regions. All the samples clearly exhibited similar IR spectra but some splits were observed after treatment with ethanol. In the IR spectrum of the human gastric mucosa before treatment with ethanol, the amide I band had one maximum at 1635 cm^{-1}, assigned to β-sheet structure, and a small shoulder at 1643 cm^{-1} attributed to random coil conformations (16, 25–26), as shown in figure 1. The amide II band exhibited a maximal peak at 1545 cm^{-1} assigned to α-helical structure. After treatment with different concentrations of ethanol for a certain period of time, however, the maximum peak of amide I band at 1635 cm^{-1} gradually disappeared and shifted to 1630 cm^{-1}, and the peak at 1641 (1643) cm^{-1} became pronounced (figs. 1 and 2). The peak at 1680 cm^{-1}, assigned to β-turn structure, also gradually decreased with the duration of ethanol treatment. This shift was faster and more pronounced for 80% ethanol than 40% ethanol. Moreover, the maximum peak of the amide II band at 1545 cm^{-1} also shifted to a higher wavenumber (1547 or 1549 cm^{-1}), while the peak at 1537 cm^{-1} due to random coil clearly appeared, but the peak at 1556 cm^{-1} assigned to β-sheet structure almost disappeared after treatment with ethanol (fig. 2). The scissoring bands also split into two peaks at 1464 and 1450 cm^{-1} as shown in figure 1. The peaks assigned to amide III (1350–1180 cm^{-1}) were changed and shifted. The peak ranging from 1180 to 980 cm^{-1}, assigned to the glycoprotein region, seemed to be lowered after treatment with ethanol.

Table 1 indicates the changes in the peak area ratio of amide I/amide II for gastric mucosa before and after treatment with ethanol (27). It clearly shows that the peak area ratio of amide I/amide II or scissoring band/amide II did not show any significant difference between gastric mucosa before and after treatment with ethanol. The peak area ratio of the COO^- band/amide II or amide III/amide II increased significantly after treatment with ethanol ($p < 0.05$). However, the peak

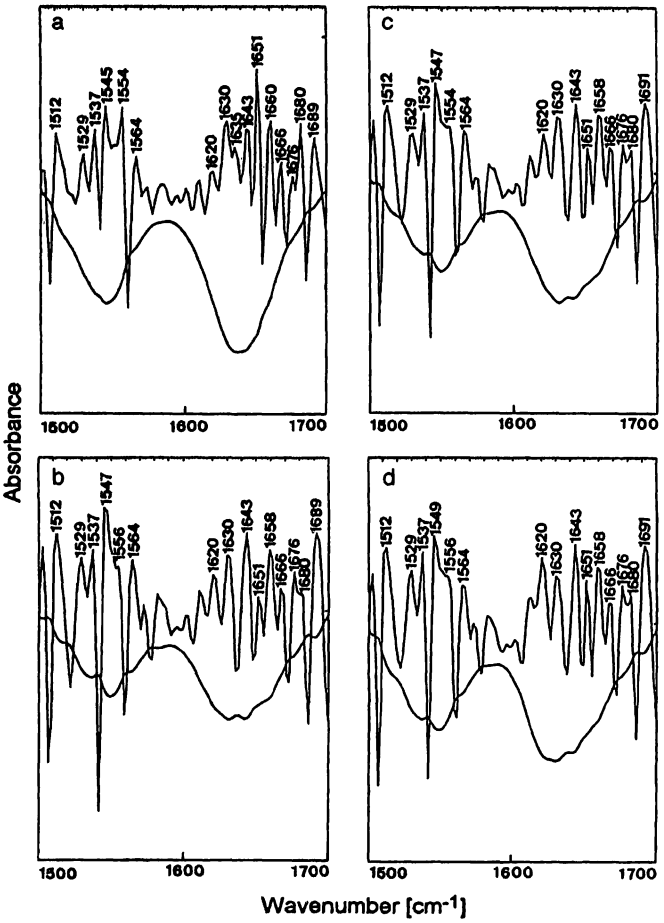

Fig. 2 Second-derivative FT-IR spectra of ethanol-treated human gastric mucosa in the 1700–1500 cm^{-1} regions
Key: (a) without ethanol treatment; (b) 40% ethanol treatment for 3 h, (c) 40% ethanol treatment for 8 h, (d) 80% ethanol treatment for 3 h

area ratio of the glycoprotein region/amide II decreased considerably with time ($p < 0.05$). Ethanol seemed to modify minor parts of the secondary structure of protein in the gastric mucosa, but it had a much greater effect on the glycoprotein structure of mucosa. The effect of ethanol concentration and time of treatment on the peak intensity ratio of each specified peak/1549 cm^{-1} for the gastric mucosa is shown in table 2. All the peak intensity ratios for the β-sheet and/or β-turn structures changed significantly, indicating modification of the conformation after treatment with 40% or 80% ethanol; the peak intensity ratios at 1689, 1622 and 1564 cm^{-1} increased, but the peak intensity ratios at 1635 and 1630 cm^{-1} decreased ($p < 0.05$). On the other hand, the peak intensity ratios at 1657 and 1545 cm^{-1} assigned to α-helical structure, and at 1641 and 1537 cm^{-1} attributed to random coil structure did not appear to be influenced by ethanol ($p > 0.05$). We also found that the peak intensity ratio at 1240 cm^{-1}, assigned to amide III, increased with the time of treatment, but the peak intensity ratio of the glycoprotein region (1082, 1055 and 1033 cm^{-1}) decreased significantly after treatment with ethanol.

Tab. 1 Peak area ratio for human gastric mucosa before and after treatment with ethanol

Treatment time (h)	Amide I* / Amide II	Scissoring / Amide II	COO^- / Amide II	Amide III / Amide II	Glycoprotein / Amide II
(A) Gastric mucosa treated with 40% ethanol					
Before treatment	1.565** ± 0.194	0.082 ± 0.003	± 0.109 ± 0.003	0.263 ± 0.018	0.449 ± 0.060
1 hour	1.732 ± 0.094	0.106 ± 0.019	0.097 ± 0.003	0.289 ± 0.014	0.234 ± 0.032
3 hours	1.769 ± 0.046	0.112 ± 0.021	0.121 ± 0.002	0.306 ± 0.048	0.298 ± 0.082
8 hours	1.638 ± 0.027	0.101 ± 0.016	0.120 ± 0.005	0.302 ± 0.009	0.286 ± 0.048
ANOVA test	NS	NS	$p < 0.05$	$p < 0.05$	$p < 0.05$
(B) Gastric mucosa treated with 80% ethanol					
Before treatment	1.565 ± 0.194	0.082 ± 0.003	0.109 ± 0.003	0.263 ± 0.018	0.449 ± 0.060
1 hour	1.643 ± 0.079	0.091 ± 0.008	0.101 ± 0.003	0.267 ± 0.013	0.215 ± 0.030
3 hours	1.584 ± 0.062	0.088 ± 0.025	0.133 ± 0.018	0.311 ± 0.015	0.266 ± 0.001
ANOVA test	NS	NS	$p < 0.05$	$p < 0.05$	$p < 0.05$

* amide I: 1700–1590 cm^{-1}; amide II: 1590–1480 cm^{-1}; scissoring band: 1480–1430 cm^{-1}; carboxylate: 1430–1350 cm^{-1}; amide III: 1350–1180 cm^{-1}; glycoprotein: 1180–980 cm^{-1}

** Mean ± S.D. (n = 3)
$p < 0.05$: significant difference
NS: no significant difference ($p > 0.05$)

Discussion

It has been pointed out that the effectiveness of the mucus barrier in vivo depends on two factors: the depth or thickness of mucus layer and the integrity of its structure (14). Mucus may be adherent or soluble. The adherent type is difficult to remove and a layer about 180 μm thick continuously covers the mucosa. Soluble mucus is composed of mucus glycoprotein which does not adhere to the mucosal surface and is removed easily by gentle washing (28). *Kerss* et al. found rapid denaturation of mucus by ethanol concentrations greater than 80% (volume fraction 0.8), and a 60–70% decrease in the thickness of the adherent mucus when rat gastric mucosa was exposed in vitro to 20–60% ethanol for 1 h at 37 °C (14). In other words, ethanol not only easily removes some mucus from the mucosa but also partially denatures the protein contained in the mucus gel or mucosa.

In the present study, the human gastric mucosa was first washed with normal saline, then immersed in different concentrations of ethanol. Since a lot of mucus was lost in normal saline and ethanol, a greater influence of ethanol on the exposed mucosa may be expected although the peak area ratio of amide I to amide II was not significantly different (tab. 1). However, table 2 clearly indicates that β-sheet and/or β-turn structure changes after treatment with ethanol, suggesting the ability of ethanol to cause minor modifications of the protein structure of the human gastric mucosa. Moreover, we also found that ethanol seemed to have no effect on the peak components of amide I and amide II assigned to amino acid side chains (arginine residues, glutamic acid residues and/or aspartic acid residues), or on the amide II components near 1517–1512 cm^{-1} attributed to α-helix, random coil structure and/or tyrosine side chains (25, 29–31). The peaks ranging from 1350 to 1180 cm^{-1} were attributed mainly to the amide III bands in which 1304 cm^{-1} was assigned to α-helix structure and 1240 cm^{-1} to disordered structure (32). In our study, 1304 and 1236 cm^{-1} appeared on the IR absorption spectra of gastric mucosa before treatment with ethanol (fig. 1a). After ethanol treatment, the peak intensity at 1304 cm^{-1} decreased and the peak at 1236 cm^{-1} shifted to 1240 cm^{-1}. Moreover, the peak intensity at 1280 cm^{-1} appeared and the peak area ratio at 1240 cm^{-1} increased. *Jakobsen* et al. mentioned that the 1240 cm^{-1} band increased upon unfolding, the 1300 cm^{-1} band decreased as protein unfolded, and the 1280 cm^{-1} band appeared after extensive unfolding of protein (32). Based on these results, we deduce that ethanol modifies the secondary structure of amide III of gastric mucosa to form a random coil structure with extensive unfolding.

It is becoming increasingly recognized that mucus glycoprotein plays a paramount role in epithelial protection (33–34). The gastric mucus glycoprotein is a covalent

Tab. 2 Peak intensity ratio for human gastric mucosa before and after treatment with ethanol

Treatment time (h)	Peak intensity ratio of the listed peak to the amide II peak at 1549 cm^{-1}													
	1689	1657	1641	1635	1630	1622	1564	1549	1545	1537	1240	1082	1055	1033
	Secondary structure*													
	(2) (3)	(1)	(4)	(2)	(2)	(2)	(2)	(1)	(1)	(4)	(4)			
(A) Gastric mucosa treated with 40% ethanol														
Before treatment	0.143** ± 0.021	1.039 ± 0.117	1.387 ± 0.119	1.412 ± 0.005	1.308 ± 0.013	0.960 ± 0.022	0.374 ± 0.041	1	1.012 ± 0.0034	0.901 ± 0.065	0.167 ± 0.017	0.324 ± 0.057	0.234 ± 0.029	0.148 ± 0.038
1 hour	0.181 ± 0.010	1.152 ± 0.065	1.396 ± 0.049	1.346 ± 0.001	1.300 ± 0.035	1.036 ± 0.034	0.620 ± 0.035	1	0.986 ± 0.024	0.879 ± 0.027	0.194 ± 0.012	0.170 ± 0.024	0.122 ± 0.017	0.086 ± 0.007
3 hours	0.250 ± 0.022	1.101 ± 0.037	1.293 ± 0.021	1.234 ± 0.047	1.252 ± 0.031	1.010 ± 0.042	0.589 ± 0.053	1	0.930 ± 0.043	0.839 ± 0.033	0.215 ± 0.007	0.176 ± 0.028	0.153 ± 0.046	0.103 ± 0.041
8 hours	0.246 ± 0.011	1.026 ± 0.033	1.255 ± 0.021	1.334 ± 0.011	1.253 ± 0.026	1.233 ± 0.049	0.530 ± 0.011	1	1.025 ± 0.022	0.962 ± 0.067	0.225 ± 0.008	0.213 ± 0.024	0.154 ± 0.029	0.092 ± 0.024
ANOVA test	$p < 0.05$	NS	NS	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$		NS	NS	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
(B) Gastric mucosa treated with 80% ethanol														
Before treatment	0.143 ± 0.021	1.039 ± 0.117	1.387 ± 0.119	1.412 ± 0.005	1.308 ± 0.013	0.960 ± 0.022	0.374 ± 0.041	1	1.012 ± 0.034	0.901 ± 0.065	0.167 ± 0.017	0.324 ± 0.057	0.234 ± 0.029	0.148 ± 0.038
1 hour	0.195 ± 0.014	1.037 ± 0.056	1.305 ± 0.034	1.342 ± 0.016	1.338 ± 0.032	1.191 ± 0.036	0.575 ± 0.044	1	0.990 ± 0.017	0.875 ± 0.044	0.211 ± 0.008	0.162 ± 0.023	0.127 ± 0.030	0.097 ± 0.017
3 hours	0.262 ± 0.027	0.951 ± 0.042	1.216 ± 0.024	1.172 ± 0.049	1.232 ± 0.032	1.161 ± 0.043	0.501 ± 0.012	1	0.947 ± 0.040	0.879 ± 0.059	0.217 ± 0.009	0.198 ± 0.009	0.143 ± 0.005	0.084 ± 0.007
ANOVA test	$p < 0.05$	NS	NS	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$		NS	NS	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

* Secondary structure: (1) α-helix; (2) β-sheet; (3) β-turn; (4) random coil
** Mean ± S.D. (n = 3)

$p < 0.05$: significant difference
NS: no significant difference ($p > 0.05$)

polymer of subunits linked by disulphide bridges, which are located in the non-glycosylated parts of the protein core, and which are susceptible to proteolysis (35). In *Takagi*'s report, 0.06–0.1 mol/l ethanol induces mucus glycoprotein synthesis, but it is still unknown whether ethanol can also adversely affect the mucus structure and what the nature of the impairment is (15). In the present study, the peak intensity ratio at 1082, 1055 or 1033 cm^{-1} in the glycoprotein groups for gastric mucosa was significantly reduced after treatment with ethanol, which agrees with the data of *Slominay* (36) in which the chemical composition of gastric mucus after pretreatment with indomethacin showed a 16% increase in protein, a 25% decrease in carbohydrate and a 53% decrease in phospholipids. The decrease of glycoproteins might also be partly due to the loss of adherent mucus from gastric mucosa after in vitro exposure to ethanol (14). The changes in the secondary structure of the isolated mucus gel and mucus-free gastric mucosa evoked by ethanol will be investigated in the future.

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Prof. Shan-Yang Lin, Ph. D.
Department of Medical Research
Veterans General Hospital-Taipei
Shih-Pai, Taipei
Republic of China